

REMARKS

Claims 1-6, 10, 16-19, 21, 23-25, 27-37, 41-45, 47 and 49-61 are pending in this application. Claims 45, 47 and 50-52 are withdrawn.

Claims 16 and 53-55 are currently amended. Support for the amendments can be found in the specification and in the original claims as filed. No new matter has been added.

WITHDRAWN RESTRICTION REQUIREMENT AND ALLOWABLE SUBJECT MATTER

Applicants gratefully acknowledge that the Examiner has withdrawn the restriction between Groups I and II. Applicants also acknowledge the indication, at page 8 of the Office Action, that claims 18, 27-32, 42-43 and 61 contain allowable subject matter. In view of the foregoing amendments and the following remarks, however, Applicants believe that all of claims 1-6, 10, 16-19, 21, 23-25, 27-37, 41-45, 47 and 49-61 are allowable.

CLAIM REJECTIONS - 35 USC § 112

At page 3, the Office Action rejects claims 53-55 as being indefinite. Applicants respectfully traverse the rejection.

Currently amended claims 53-55 address each of the issues noted in the Office Action. In particular, amended claims 53 and 54 depend from claim 17, and claim 17 provides antecedent support for size-discriminating ultrafiltration and ultracentrifugation. Amended claim 55 now depends from claim 1.

Also, not mentioned in this rejection, amended claim 16 now depends from claim 1. Accordingly, Applicants request reconsideration and withdrawal of the rejection.

CLAIM REJECTIONS - 35 USC § 103

At page 4, the Office Action rejects claims 1-6, 16, 19, 21, 25, 33-37, 44, 49, 56-58 and 60 under 35 U.S.C. § 103(a) as being unpatentable over KAUTIAINEN et al., (A liquid chromatography tandem mass spectrometric method for in vivo dose monitoring of diepoxybutane, a metabolite of butadiene, *Rapid Communications in Mass Spectrometry*, 14 (2000) p. 1848-1853), further in view of RYDBERG et al. (Adducts to N-terminal valines in hemoglobin from butadiene metabolites, *Chemico-Biological Interactions*, 101 (1996) p. 193-205).

At pages 6-8, the Office Action rejects claims 10, 17, 24, 41 and 59 under 35 U.S.C. § 103(a) over KAUTIAINEN and RYDBERG, and further in view of TORIBA et al. (Development of an Amino Acid Sequence and d/l-Configuration Determination Method of Peptide with a New Fluorescence Edman Reagent, 7-Methylthio-4-(2,1,3-benzoxadiazolyl) Isothiocyanate) *Anal. Chem.* 72 (2000) p. 732-739).

Applicants respectfully traverse these rejections.

The present claims are directed to methods for analyzing N-adducted amino acids or adducted N-terminal peptide/proteins. The method utilizes an isothiocyanate reagent

containing a fluorescent moiety and an ionizable moiety - FITC, DNITC or DABITC. The N-terminal adducts are then detected using liquid chromatography and mass spectrometry. KAUTIAINEN and RYDBERG fail to teach or suggest this method.

The art of measuring N-terminal protein adducts by use of Edman isothiocyanate reagents is a rather specialized field in science. Measurements of such adducts are normally performed at extremely low adduct levels (i.e. one modified N-terminal amino acid out of 100,000 to 10,000,000 normal/unmodified terminal amino acids). Consequently, the present application must be compared with other analytical methods in the same research field, e.g., adduct measurements on N-terminal amino acids in proteins, and not with methods conducting detection and analysis of amino acid sequences. Existing methods in the art have utilized either phenyl isocyanate (PIC) or pentafluorophenyl isothiocyanate (PFPITC), which are non-ionizable and non-fluorescent isothiocyanate reagents. An extensive background of the N-alkyl Edman method has been given in the present specification. (See, Introduction and Refs. 1 - 18).

The present patent application describes several advantages of using ionizable and fluorescent isothiocyanate reagents for measurements of N-terminal adducts. A few examples from the specification are that this new approach provides the possibility to selectively enrich (e.g., by ion exchangers) and to selectively measure N-terminal secondarily bound adducts

(e.g. with LC-MS/MS)) with high sensitivity (see, Table 1, and Figures 17 and 18). The FIRE-procedure (fluorescent/ionizable N-R-Edman procedure) can be utilized for direct measurements in complex mixtures, such as whole blood, without previous purification of the desired proteins. The introduction of reagents that contain ionizable groups speeds up the analytical procedure as the number of analytical steps and "hands on time" has been reduced considerably (see, paragraph [0151] and Figure 19). These advantages are hereafter referred to as "benefits with the FIRE procedure" where the FIRE-procedure is the method described in the present specification and is abbreviated from fluorescent/ionizable N-R-Edman procedure.

In KAUTIAINEN, a method was introduced to measure ring closed pyrrolidine N-terminal protein adducts formed from diepoxibutane. After enzymatic digestion (trypsin) the N-terminal pyrrolidine adduct was bound to a heptapeptide and measured by LC-MS/MS. KAUTIAINEN discloses the utilization of enzymatic degradation followed by LC-MS/MS analysis of trypsinated protein-bound N-terminal adducts. In contrast to the presently claimed method, however, KAUTIAINEN fails to teach or suggest fluorescent and ionizable Edman reagents, such as FITC, to measure the N-terminal protein adducts.

Indeed, the adduct measured in KAUTIAINEN is a tertiary bound pyrrolidine adduct, therefore, it cannot be detached with an isothiocyanate Edman reagent. Isothiocyanate Edman reagents do

not react with tertiary amines. The presently claimed method utilizes fluorescent and ionizable isothiocyanate reagents and not PITC or PFPITC. The difficulties with the sensitivities of PITC and PFPITC analytes in LC-MS/MS are discussed above.

In RYDBERG, a method was introduced to measure N-substituted adducts on the amino acid valinamide, used as a model for N-terminal valine in hemoglobin. The proteins were reacted with diepoxibutane and a number of adducts were identified. Secondary bound adducts such as N-(2,3,4-trihydroxybutyl)valinamide could be measured after derivatisation with the Edman reagent PFPITC. As the ring closed, tertiary bound pyrrolidine adduct could not be measured by the N-alkyl Edman method and extensive work was initiated which resulted in the methods described in the KAUTIAINEN reference above.

RYDBERG utilizes the N-alkyl Edman method, with the reagent PFPITC, for measurement of secondary-bound adducts to model compounds for N-terminal valine in hemoglobin. In distinction from the presently claimed methods, no fluorescent and ionizable Edman reagent, such as FITC, was used to study or measure the N-terminal protein adduct.

The combination of KAUTIAINEN and RYDBERG fails to teach or suggest anything to guide one of ordinary skill in the art to solve the problems solved by the present application (as described above). One of ordinary skill would not combine these

papers. The method described in RYDBERG is not applicable for the tertiary pyrrolidine adduct measured in KAUTIAINEN. The present application solves many problems and the unexpected high sensitivity on LC-MS/MS would not be foreseen by one of ordinary skill in the art.

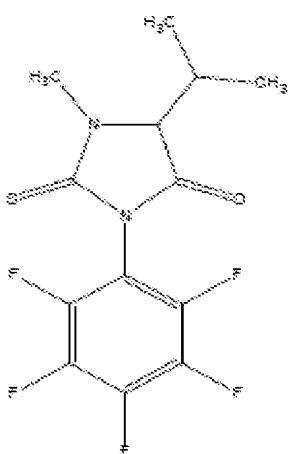
The sensitivity when measuring the analytes studied in KAUTIAINEN, due to the lack of ionizable groups in the utilized Edman reagent PFPITC, will be much lower when measuring with LC-MS/MS than with GC-MS/MS (the used method). This large difference in sensitivity was unexpectedly found when comparing different sensitivities on LC-MS/MS with N-methylated valine thiohydantoines formed from FITC, DABITC, DNITC, PITC and PFPITC (see, Table 1). One of ordinary in the art would find that LC-MS/MS analysis when using PFPITC would be very low on LC-MS/MS, much lower than with GC-MS/MS (as PFPITC is known to provide excellent sensitivity by this method of analysis). The idea to combine KAUTIAINEN with RYDBERG will only lead one of ordinary skill to draw the conclusion that LC-MS/MS analysis of PFPITC analytes gives such poor sensitivity to be of no value. For all of these reasons, the combination of KAUTIAINEN and RYDBERG fail to teach or suggest, and would not have rendered obvious, claims 1-6, 16, 19, 21, 25, 33-37, 44, 49, 56-58 and 60.

In further regard to claims 6 and 37, and the featured serum albumin adducts, it is extremely difficult to obtain measurements of serum albumin adducts by the N-alkyl Edman

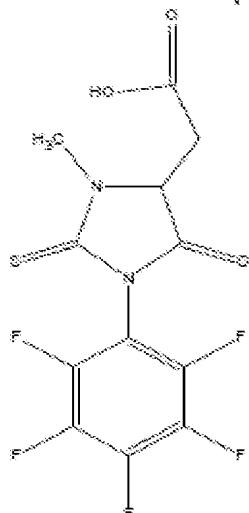
method. Despite that the N-alkyl Edman method was published in 1986 and that it is utilized at over 20 laboratories worldwide, there has not come out one single document where this method is used for measurements of adducts to N-terminal aspartic acid in serum albumine. The reasons for this are as follows.

First, the N-alkyl Edman method was developed for adduct measurements on isolated haemoglobin Hb and not for serum albumine (SA), thus, the methods must be modified. Second, the side chain of aspartic acid makes the formed SA analyte extremely hydrophilic as the analyte will be negatively charged at pH 7 which is the pH used for extraction of the analyte. This second modification is difficult due to the differences in solubility of PFPTH-MeVal and PFPTH-MeAsp (see, figure presented below). At pH 7, there is a calculated difference in the solubility system octanol water of a factor of over 4 magnitudes (log 3.92 to 0.54). One could argue that the extraction could be done at pH 2. This, however, would not work out as acids catalyze ringclosure of unmodified (normal) N-terminals (Edman degradation conditions) and the signal from low levels of N-modified analyte would formally drown. Finally, to run PFPTH-MeAsp on GC-MS would require prior derivatisation, e.g, methylation of the carboxylic acid, which is another tricky step that must be developed and evaluated.

PFPTH-MeVal



PFPTH-MeAsp



Solubility at 25°C in water, S (Tm: 210°C):

pH	log(1/S) [± 1.0]	Solubility, g/L
0.0	3.88	0.044
1.0	3.92	0.041
2.0	3.92	0.041
3.0	3.92	0.041
4.0	3.92	0.041
5.0	3.92	0.041
6.0	3.92	0.041
7.0	3.93	0.041
8.0	3.92	0.041
9.0	3.92	0.041
10.0	3.92	0.041
11.0	3.92	0.041
12.0	3.92	0.041
13.0	3.92	0.041
14.0	3.92	0.041

Solubility at 25°C in water, S (Tm: 210°C):

pH	log(1/S) [± 1.0]	Solubility, g/L
0.0	2.59	0.91
1.0	2.59	0.91
2.0	2.59	0.92
3.0	2.53	1.05
4.0	2.19	2.30
5.0	1.38	14.8
6.0	0.41	138
7.0	-0.54	1,241
8.0	-1.25	inf infinitely Soluble
9.0	-1.47	inf infinitely Soluble
10.0	-1.50	inf infinitely Soluble
11.0	-1.51	inf infinitely Soluble
12.0	-1.51	inf infinitely Soluble
13.0	-1.51	inf infinitely Soluble
14.0	-1.51	inf infinitely Soluble

In conclusion, the methods of KAUTIAINEN and RYDBERG are not applicable for measurements of SA adducts. In contrast, the FIRE-procedure of the present claims is suitable as analytes from proteins or whole blood can be purified (e.g., with ultracentrifugation and ion-exchangers) and the method does not discriminate ionic and polar analytes. For these additional reasons, KAUTIAINEN and RYDBERG would not have rendered obvious claims 6 and 37.

In regard to the rejection of claims 10, 17, 24, 41 and 59, TORIBA describes a fluorescent Edman reagent, 7-methylthio-4-(2,1,3-benzoxadiazolyl) isothiocyanate (MTBD-NCS) to measure the sequence of amino acids in proteins. TORIBA, however, does not use the MTBD-NCS reagent to measure N-terminal protein adduct in proteins. TORIBA utilizes fluorescent Edman reagents for sequencing proteins (i.e., measuring amino acid thiohydantoines lacking adducts). As discussed above, the chemistry dealing with sequencing proteins has very little in common with methods described for measuring N-terminal adducts. TORIBA fails to disclose anything that would allow one of ordinary skill in the art to realize any benefit provided by utilizing fluorescent and ionizable isothiocyanate reagents for adduct measurements, as featured in the claimed methods.

For these additional reasons, KAUTIAINEN, RYDBERG and TORIBA fail to teach or suggest the method of claim 10, 17, 24, 41 and 59. Accordingly, Applicants request reconsideration and withdrawal of the rejection.

CONCLUSION

Entry of the above amendments is earnestly solicited. Applicant respectfully requests that a timely Notice of Allowance be issued in this case.

Should there be any matters that need to be resolved in the present application the Examiner is respectfully requested to contact the undersigned at the telephone number listed below.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

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